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Use of immobilized synthetic peptides for the identification of contact sites between human interleukin-6 and its receptor

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Abstract Synthetic peptides immobilized on cellulose membranes proved to be a powerful tool for the identification of sites in the cytokine IL-6 involved in receptor binding. Similarly, a region in the extracellular part of the IL-6 receptor which is important for interaction with its ligand was identified.

Key words: Cellulose-bound synthetic peptide; Peptide scan; Interleukin-6; Interleukin-6 receptor; Protein–protein interaction

1. Introduction

Protein–protein interactions have been studied by a multitude of methods. Crosslinking experiments, competition studies with monoclonal antibodies, site-directed mutagenesis, X-ray crystallography, fluorescence-, mass- and NMR-spectroscopy have been powerful approaches.

Since we have been interested in structure–function studies on the cytokine interleukin-6 and its receptor for several years [1–6], we have now applied the use of synthetic peptides covalently linked to cellulose membranes [7,8] in order to identify contact sites between IL-6 and its receptor.

IL-6 exerts pleiotropic activities in hematopoiesis, in the immune response and in the acute phase reaction of the liver [9–11]. These functions are mediated via a two-component cell surface receptor complex consisting of a ligand binding α subunit (gp80, IL-6R) and a signal transducing protein (gp130) [12].

IL-6 is known to belong to a family of hematopoietic cytokines adopting an α-helix bundle structure. The four major α-helices of the molecule are arranged in an up-up-down-down topology [13]. The IL-6 receptor, on the other hand, is a member of the immunoglobulin superfamily, i.e. its extracellular part consists of one distal IgG-like domain and two proximal fibronectin type III modules constituting an IL-6 binding pocket and thus referred to as a cytokine binding domain [14].

The receptor interaction site of IL-6 appears to be made up of crucial residues in helix A, in the C-terminal part of the AB-loop and at the end of helix D [1–3,5,15–18]. The corresponding surface on the IL-6 receptor is formed by residues in the B′C′-loop and adjacent C′-strand as well as in the D′E′- and F′G′-loops of the second cytokine binding domain ([19]; M. Kalai et al., submitted for publication).

In the work presented we were able to identify two regions in the IL-6 molecule and one region in the IL-6 receptor which form contact sites in the ligand–receptor complex using immobilized synthetic peptide fragments of the two proteins.

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Abbreviations: IL-6, interleukin-6; IL-6R, interleukin-6 receptor; sIL-6R, soluble IL-6R.

2. Materials and methods

2.1. Iodination of human IL-6 and sIL-6R

Human recombinant IL-6 was expressed in *E. coli* and purified from inclusion bodies as described in [20]. A specific activity of 10^8–10^9 units per mg of protein was determined in the B9 cell proliferation assay [21]. Soluble IL-6R was purified to homogeneity from conditioned media of baculovirus-infected Sf9 cells by affinity chromatography [22]. Iodination of both probes was performed according to [23]. Specific activities of 0.5 MBq/μg and 0.8 MBq/μg were obtained for IL-6 and sIL-6R, respectively.

2.2. Synthesis of peptides immobilized to cellulose membranes

The cellulose-bound peptides were prepared automatically according to standard spot synthesis protocols [7] using a spot synthesizer (Abimed GmbH, Langenfeld, Germany) as described in detail previously [8].

2.3. Probing of membrane-bound peptides for [^{125}I]sIL-6R- or [^{125}I]IL-6 binding

Membranes were rinsed with methanol and washed three times with TBS (137 mM NaCl, 2.7 mM KCl, 50 mM Tris-HCl, pH 8.0). To prevent unspecific binding the membranes were incubated overnight in blocking buffer (Cambridge Research Biochemicals, Gadbrook Park, UK) containing 0.15 M sucrose, 0.05% Tween 20 and 0.01% NaN_3. Following another washing step in T-TBS (TBS containing 0.05% Tween 20), membranes were assayed for binding activity by incubation with the interacting ^{125}I-labelled protein in blocking buffer for 3 h at room temperature. After three washes in T-TBS, bound radioactivity was visualized by exposure to an X-ray film for 24–72 h.

3. Results

In order to analyze the sites of interaction between IL-6 and its α receptor subunit (IL-6R), dodecapeptides spanning the whole IL-6 sequence and overlapping by nine amino acids were synthesized on a cellulose membrane support. A soluble form of the IL-6R, which we have expressed in baculovirus-infected insect cells, was radiolabelled with ^{125}I and used to probe the membrane for binding activity. Bound radioactivity was visualized by autoradiography (Fig. 1A). Binding of [^{125}I]sIL-6R to IL-6 peptides essentially focuses on two distinct regions com-

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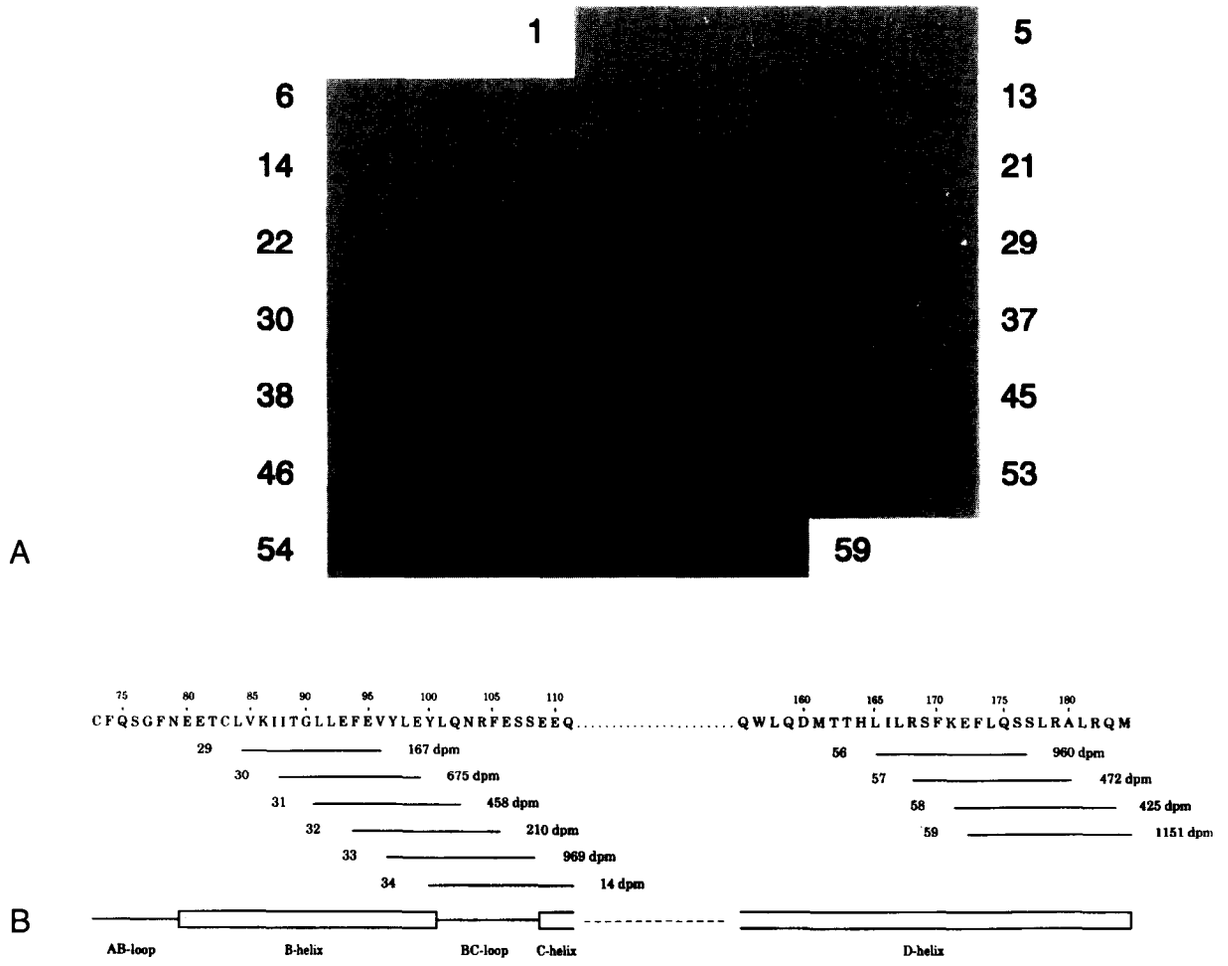

Fig. 1. Spotscan of membrane-bound IL-6-derived dodecapeptides with [^{125}I]sIL-6R. (A) 59 dodecapeptides spanning the entire IL-6 amino acid sequence were synthesized as described in section 2. Peptides on adjacent spots differ by a shift of three amino acids from each other, i.e. they overlap by nine amino acids. Numbers denote the first and last spot in each row. The membrane was incubated with 20 ng of [^{125}I]sIL-6R (specific activity 0.8 MBq/μg) per ml of blocking buffer. After extensive washing, bound radioactivity was visualized by autoradiography. (B) Schematic representation of the amino acid sequence (top) and the predicted secondary structure elements (bottom) of IL-6 [13] corresponding to peptides #29 to #34, and #56 to #59. The radioactivity on each spot (background subtracted) is indicated.

prised in the peptides #30 to #33, and #56 to #59, respectively. The location of these overlapping peptides within the sequence of IL-6 and predicted secondary structure elements [13] are indicated in Fig. 1B, lower part; the first of the two regions of interest (I_{88}–S_{108}) is localized at the C-terminal part of helix B and the adjacent BC-loop, whereas the second one (L_{165}–M_{184}) corresponds to the C-terminus of the molecule.

To further characterize these two regions with respect to the residues essential for binding, mutation experiments were performed with dodecapeptides G_{90}–L_{101} and F_{173}–M_{184}. Each residue was separately exchanged for each of the 20 amino acids. The results are shown in Fig. 2. In the G_{90}–L_{101} peptide (Fig. 2A), all three glutamic acids are obviously essential for binding of [^{125}I]sIL-6R, since any substitution except for aspartic acid abolishes binding. Introduction of additional negative charges (E or D) in many positions increases bound radioactivity above the level of the native peptide, most impressively at positions L_{92}, Y_{97}, L_{98} and Y_{100}. On the other hand, V_{96} does not tolerate exchanges for charged amino acids; hydrophobic or small polar side chains are preferred at this position. In the second peptide mutated (F_{173}–M_{184}) (Fig. 2B), R_{179} and R_{182} are least tolerant towards mutations, which reflects their crucial role in [^{125}I]sIL-6R binding. All substitutions except for K decrease or abolish binding of labelled IL-6 receptor. Thus far, we and others were unsuccessful in solving the 3D structure of IL-6 either by NMR spectroscopy or X-ray crystallography. A model of IL-6 has been built with the X-ray structure of G-CSF [24] as a template [5]. The regions of IL-6 (black) which we found to be involved in receptor binding are shown in the model of the IL-6/IL-6R complex (Fig. 4, left part).

An analogous approach was used to elucidate the ligand binding epitopes on the IL-6R molecule: membrane-bound tridecapeptides derived from the two ligand binding domains of the IL-6R were scanned with [^{125}I]IL-6 (Fig. 3A). Bound radioactivity was mainly detected in one cluster of spots (#80 to #87). Fig. 3B shows the localization of this epitope within the primary and predicted secondary structure of the IL-6R [14]. The region of interest comprises residues W_{219}–K_{252} and

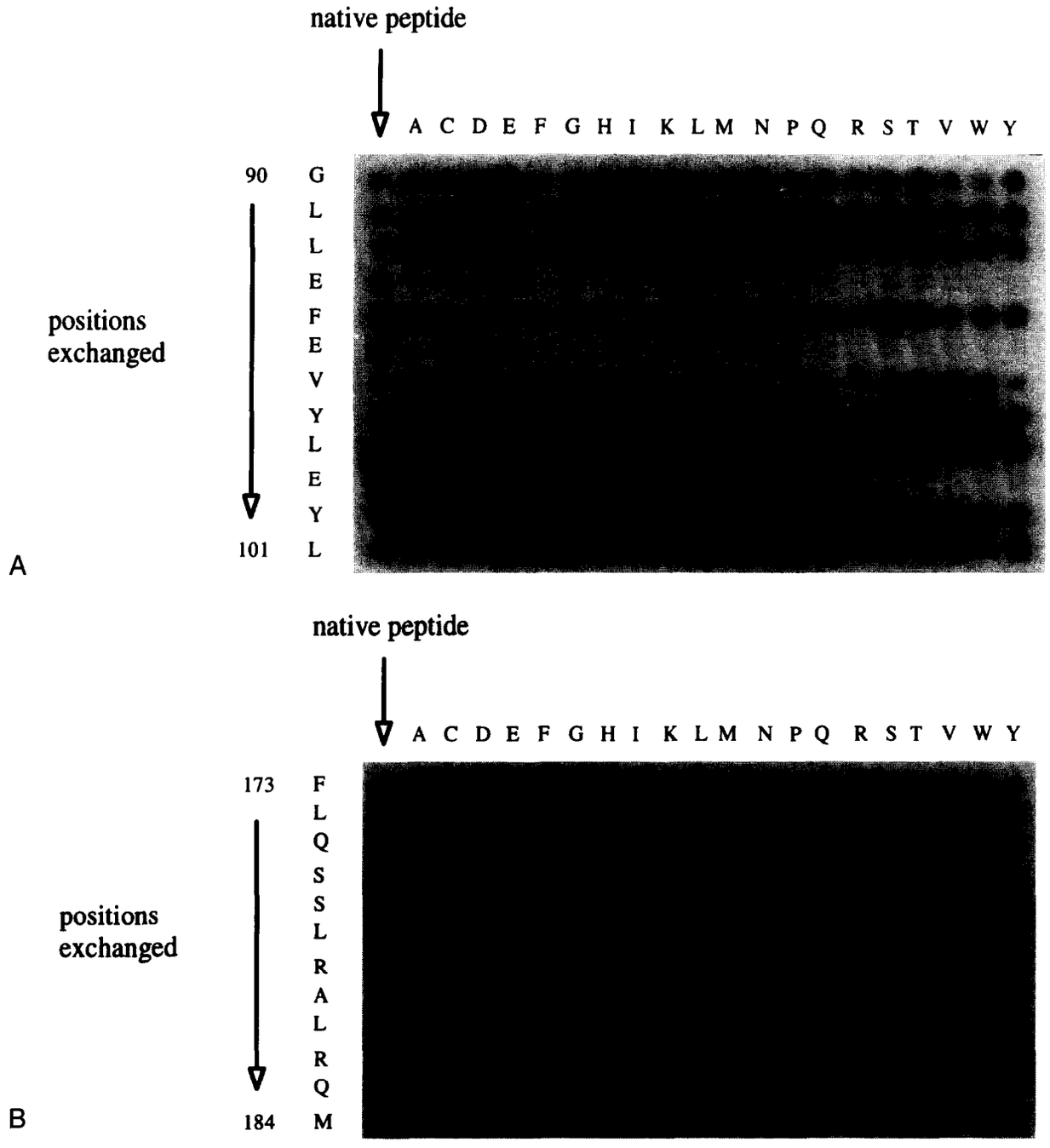

Fig. 2. Spotscan of point-mutated IL-6 peptides with [^{125}I]sIL-6R. In peptides G_{90}–L_{101} (A) and F_{173}–M_{184} (B) defined in Fig. 1 each residue was separately exchanged by each of the 20 proteinogenic amino acids. The positions mutated are indicated at the left, the amino acids inserted instead are shown on top of the autoradiography. The membranes were probed for [^{125}I]sIL-6R binding, as in the previous experiment.

extends over β-strands C′ and D′ and the B′C′- and C′D′-loops. This stretch on the IL-6R molecule is marked in black in Fig. 4, right part.

4. Discussion

In this study, an approach using immobilized synthetic peptides was used to investigate the protein–protein interaction in a cytokine-receptor system. Applying this technique to human IL-6 and its receptor, we were able to confirm results obtained by other methods: the extraordinary importance of the C-terminus of IL-6 for IL-6R binding, especially of R_{179} and R_{182} [2,3,16–18] is clearly demonstrated by our results. The second IL-6 region that appears to be involved in the interaction with IL-6R (end of B-helix, BC-loop) has also been described before [25]. In this region, our point mutational scan suggests a major role of three glutamic acid residues in IL-6R binding. Parts of helix A and of the AB-loop have been identified to be involved

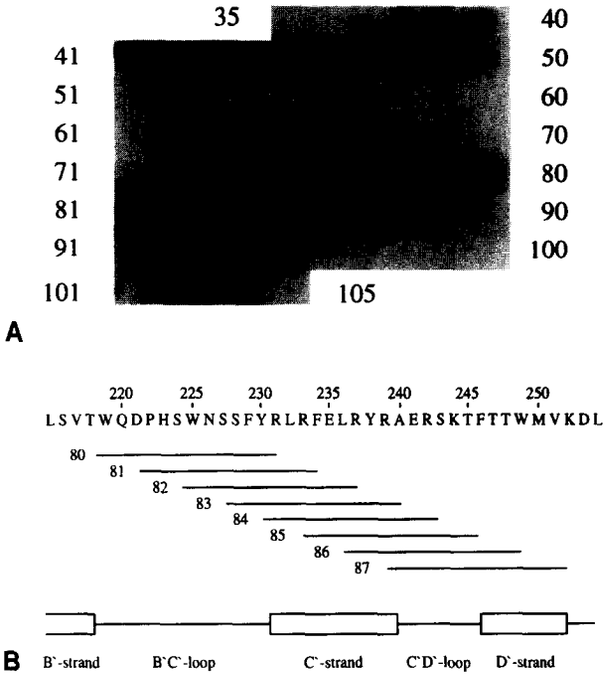

Fig. 3. Spotscan of sIL-6R-derived tridecapeptides with [^{125}I]sIL-6. (A) Membrane-bound tridecapeptides overlapping by ten amino acids were probed with [^{125}I]IL-6 (30 ng/ml) specific activity 0.5 MBq/μg), followed by extensive washing and autoradiography. (B) Schematic mapping of peptides #80 to #87 to the primary (top) and predicted secondary structure (bottom) of the IL-6R [14].

in IL-6R binding [5,26]. These regions within the IL-6 molecule could not be detected by our spot scan technique, probably because they are parts of discontinuous epitopes. Although the position of the IL-6R in our model (Fig. 4) is validated by our results (Fig. 1, end of helix D) and by various experimental data [3,5,15,26], our results on the BC-loop interaction of IL-6 with the IL-6R may suggest that the model shown in Fig. 4 is incomplete. Consequently, a second IL-6R molecule may interact with this region.

In the IL-6R molecule, we found the stretch of residues W_{219}–K_{252} implicated in IL-6 binding. With respect to the three-dimensional topology illustrated in Fig. 4, right part, involvement of such an extended region in ligand binding seems rather unlikely, since the end of strand C′, the C′D′-loop and strand D′ are not exposed to the ligand binding surface as presumed on the basis of a molecular modelling approach using the crystal structure of the human growth hormone/receptor complex [27]. Point mutations in the IL-6R molecule revealed the importance of Y_{230} and R_{231} for ligand interaction ([19]; M. Kalai et al., submitted for publication). Among the peptides with IL-6 binding activity, #80 to #85 at least partly comprise these crucial residues.

The positive signal from the peptides #86 and #87 is most probably due to the presence of two motives ($\mathbf{R}_{237}\mathbf{YRAE}_{241}$ and $\mathbf{R}_{242}\mathbf{SKTF}_{246}$) mimicking the $\mathbf{R}_{231}\mathbf{LRFE}_{235}$ sequence in the IL-6 binding pocket. Obviously, the presence of two positively charged amino acids with one uncharged residue in between is sufficient for IL-6 binding. Grube et al. [28] used soluble synthetic peptides, which are also comprised by the IL-6R region identified in this study, in order to evaluate their interference with ligand binding and signal transduction. They found peptide Y_{230}–T_{245} to inhibit biological effects of IL-6, such as acute phase protein synthesis in HepG2 cells and B9 cell proliferation. They did, however, not detect any impairment of IL-6 binding to U266 cells, which should have been expected from the results presented here. This discrepancy might be due to the higher sensitivity of our approach concerning the detection of protein–protein interaction. A soluble peptide starting at Y_{230} displays the putative IL-6 binding residues at the N-terminus. Moreover, the N-terminal Y_{230}, that obviously is involved in the interaction with IL-6, is associated with the positive charge of its underivatized amino group. Both factors might lead to reduced affinity for the ligand due to conformational problems. On the other hand, in the microenvironment at the cellulose membrane surface, synthetic peptides are present at extremely high concentrations resulting in a detectable ligand *avidity* in spite of a relatively low physical *affinity*. Other loops (D′E′ and F′G′) in the IL-6R molecule involved in the interaction with IL-6 [19] could not be identified by our approach.

Our results, of course, do not rule out a potential role of residues further downstream in the IL-6R sequence, e.g. in strand C′ or the C′D′-loop, in the interaction with gp130. Although we were able to find peptides in IL-6 as well as in the IL-6 receptor important for ligand–receptor interaction, the results obtained by this technique have to be interpreted with caution. Discontinuous epitopes are hard to detect by an approach using linear (and relatively short) peptides instead of the intact protein. Moreover, regions of a molecule that are not surface-exposed in the native structure will be displayed on the spot membrane and may therefore evoke positive signals although they are not implicated in protein–protein interaction.

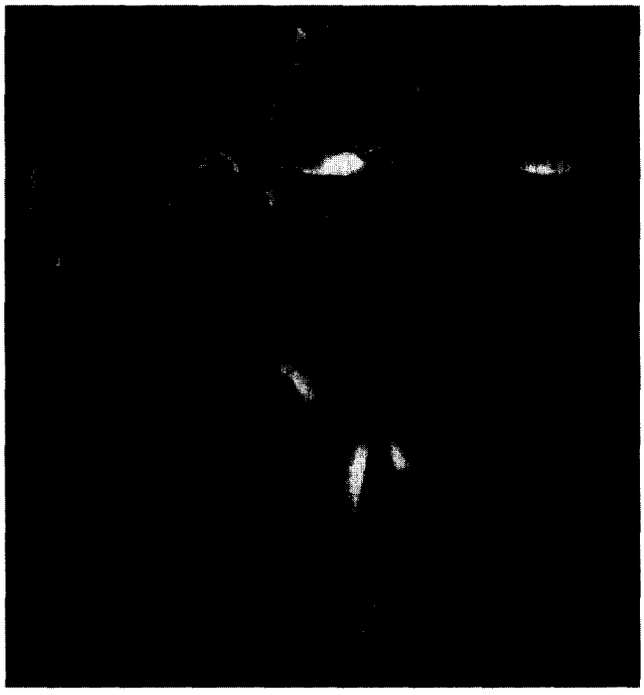

Fig. 4. A ribbon representation of a model of the IL-6/IL-6R complex which was built using the hGH/hGHR complex as a template ([5,27]; Grötzinger, J. et al., submitted for publication). Binding sites on the IL-6 (left) and IL-6R (right) molecules identified in this study are shown in black. The 4 helices of IL-6 are denoted A, B, C, D.

Nonetheless, the peptide scan approach is a fast and relatively simple screening tool that can provide valuable information on ligand–receptor interaction sites.

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